Molecular Stock Authentication

Sample preparation:

- Either adults or pupae can be used to authenticate a stock. Pupae are easier to collect, but care must be given to remove all water from the sample before processing.
- **For adults**: Collect up to 50 adults by aspirating and releasing them into a disposable 50 ml blue cap tube on ice. Cap tube immediately and chill. Process mosquitoes when immobilized. Place a 48-well PCR plate (Robbins Scientific 1055-20-0) on ice. Label plate appropriately. With feather-tip forceps, carefully place one mosquito per well. Be careful not to transfer legs or antennae from one well to the next. If necessary, wipe the forceps with ethanol and a kimwipe between mosquitoes. Leave one or two wells empty for a negative control.
  For pupae: Collect 24-48 pupae in a cup.
  Place a 48-well PCR plate (Robbins Scientific 1055-20-0) on ice. Label plate appropriately. With a pupa pipettor, carefully place one pupae into each well. Leave one or two wells empty for a negative control. Using a P-1000, carefully remove all water from each sample.
- Using a repeat pipettor, add 50 ul of Engels buffer + Triton X [1] to each well, including the negative control empty wells. Check that all the mosquitoes are at the bottom of the plate and are in buffer.
- Seal the plate carefully with half of an Adhesive Sealing Foil (Marsh AB-0626). Check that all tubes are sealed properly to avoid evaporation of sample.
- Incubate samples at 95ø for 15 min. in the thermal cycler.

Sample PCR:

- While the samples are incubating, prepare enough PCR Master Mix for 52 PCR reactions, as follows:

  For *An.gambiae* complex stocks (including, G3, ASEMBO1, AHERO, BAMAKO):

942 ul	dH2O
130 ul	10X PCR Buffer
130 ul	dNTP mix (2mM)
130 ul	GA primer (6.25 ng / 25 ul rxn)
130 ul	UN primer (12.5 ng / 25 ul rxn)
130 ul	AR primer (18.75 ng / 25 ul rxn)
15.6 ul	MgCl2 (25mM)
10.4 ul	Taq DNA polymerase (5U/ ul)

Primers should be prepared to the following concentrations (10X concentration):

GA	2.5 ng / ul (0.0025 ug / ul)
UN	5.0 ng / ul
AR	7.5 ng / ul

For *An. gambiae white* stocks (including M2 and M5):

1004 ul	dH2O
130 ul	10X PCR buffer
130 ul	dNTP mix (2mM)
26 ul	primer 87 (straight from stock)
26 ul	primer 88 (straight from stock)
15 ul	Taq DNA Polymerase (5U/ ul)

For *An. quadrimaculatus* stocks (including ORLANDO, GOCUT, Q2, RO):

806 ul	dH2O
130 ul	10X PCR Buffer
130 ul	2mM dNTP mix
104 ul	5A primer (10pmol/ ul)
104 ul	3A primer (10pmol/ ul)
26 ul	Taq DNAa Polymerase (5U/ ul)

- Place a new 48-well PCR plate on ice. Label it to match the sample plate.
- With a repeat pipettor, carefully add 25 ul of Master Mix to each well of the plate. Cover with parafilm until ready to use.
- When sample incubation is finished, remove aluminum foil carefully so as not to contaminate samples with each other. Using a sterile cell replicator (Boekel 140500), carefully transfer sample to PCR plate by dipping replicator straight down into sample plate, then dipping replicator in PCR plate. Check orientation of transfer to ensure sample transferred corresponds to correct PCR reaction. Store left-over sample at 80oC.
- Seal PCR plate with Adhesive Sealing Foil, assuring all wells are sealed completely.
- Cycle using the following cycling conditions for Perkin Elmer GeneAmp PCR System 9600

For *An. gambiae* complex PCR:

94° C/ 5min -0- (94° C/30sec -0- 50° C/30sec -0- 72° C/30sec) x 30 cycles -0- 72° C/5min -0- 4° C

For *An. quadrimaculatus* complex PCR:

94° C/ 5min -0- (94° C/30sec -0- 55° C/30sec -0- 72° C/30sec) x 35 cycles -0- 72° C/5min -0- 4° C

For *An. gambiae white* PCR:

94° C/ 5min -0- (94° C/30sec -0- 50° C/30sec -0- 72° C/30sec) x 30 cycles -0- 72° C/5min -0- 4° C

Visualization of PCR results:

- Prepare a 2% (w/v) centipede agarose gel in 1X TBE buffer by adding 200mls buffer to 4.0g agarose in a 500ml Erlenmeyer flask.
- Dissolve agarose by heating in microwave oven. Heat agarose mixture slowly and carefully to avoid boiling over.
- Cool solution to 55^0C and add 10 ul EtBr solution.
- Pour gel mixture into gel tray with 50-well comb and let gel set 40min to 1hr. When gel has hardened, overlay with 1-liter 1X TBE buffer in horizontal gel electrophoreses apparatus. Add 10 ul EtBr to lower chamber.
- Using a multichannel pipettor, add 5 ul Orange G Dye[2] by piercing through the aluminum seal with pipette tips. Carefully mix by pipetting up and down.
- Load 10 ul in gel with multichannel pipettor paying close attention to loading order.
- Visualize by placing on UV box. Photograph and store image as instructed in Storing Images SOP. Print picture and tape to data sheet.
- Place data sheet in Molecular Authentication Notebook. Enter data into database.

To clean the replicator:

- Immediately after transfer, dip replicator in a Liquinox / H_2O solution for a few hours. Scrub replicator teeth carefully to remove any left over mosquito debris.
- Rinse replicator thoroughly with dH_2O. Allow to air dry.
- Sterilize by ethanol flaming teeth.
- Wrap in clean aluminum foil until ready to use.

Notes

1 Engels buffer: 10mM Tris-HCl pH8.2, 1mM EDTA, 50mM NaCl, 0.1% TritonX-100

2 6X Loading Dye: 350mg of Orange G, 30g of sucrose, raise to a final volume of 100ml with TE. Store at 4ºC.